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Differential Action of Bacterial Phosphatidylinositol-Specific Phospholipases C on Bovine Erythrocyte Membrane Ghost

KEYWORDS: phosphatidylinositol-specific phospholipase C, glycosylphosphatidylinositol, bovine erythrocyte membrane ghost, electrophoretic profile, released proteins

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ABSTRACT *In recent years, much attention has been focused on identifying the glycosylphosphatidylinositol (GPI) anchors of eukaryotic membrane proteins and understanding their role in protein function. Commercial preparation of phosphatidylinositol-specific phospholipase C (PIPLC) from Bacillus cereusis routinely used as a tool for releasing and identifying GPI-anchored proteins (GPI-AP). Here, we compared the action of PIPLC from Bacilluscereus(Bc-PIPLC)and Staphylococcus aureus (Sa-PIPLC) on bovine erythrocyte membrane ghost (BEMG). BEMG treated with Bc-PIPLC or Sa-PIPLC showed dissimilar electrophoretic pattern of released GPI-APs.The result indicates that the resistance and susceptibility of GPI-APs to PIPLC depends on the source of enzyme and this aspect warrants serious consideration while using PIPLC as a tool for identification of GPI-APs. Further, combination of PIPLCs from two sources showed a cumulative effect in releasing GPI-APs suggesting it to be more efficient in identification of GPI-APs than the conventional use of a single PIPLC preparation.*

INTRODUCTION

Phosphatidylinositol-specific phospholipase C (PIPLC), an important class of phospholipases is produced by many bacterial species, protozoa, yeasts, plants and mammals (Eisanhaber et al., 2001). Eukaryotic PIPLCs are involved in signal transduction processes and bacterial PIPLCs are known virulence factors. In addition to hydrolyzing phosphatidylinositol (PI), the enzyme also hydrolyses glycosylphosphatidylinositol (GPI) (Ikezawa, 2002). GPI is one of the unique anchoring modes of certain important eukaryotic membrane proteins such as alkaline phosphatase and acetylcholinesterase. In recent years, much attention has been focused on identifying the glycosylphosphatidylinositol (GPI) anchors of eukaryotic membrane proteins and understanding their role in protein function. As bacterial PIPLCs release GPI-anchored proteins (GPI-APs) from the eukaryotic cell surfaces they are routinely used for identification of GPI anchor (Ikezawa, 2004; Paulick and Bertozzi, 2008). Release of membrane proteins with PIPLC is a powerful criterion for GPI anchor identification. An alternative is to predict GPI anchor using bioinformatics approaches that recognize the C-terminal GPI-anchoring signals (Eisanhaber et al., 2003). However, with the *in silico* GPI anchor prediction tools, it is difficult to balance between false positive and false negative errors (Fankhauser and Maser, 2005). As a result, these prediction tools also require experimental validation by treatment with PIPLC.

In view of the potential role of PIPLC in GPI-related studies, there is an urgent need to explore PIPLC from different sources. In the present study, we compared the action of two bacterial PIPLCs on bovine erythrocyte membrane ghosts (BEMG). Electrophoretic profile of released proteins indicated the differential action of the two PIPLCs in releasing the GPI-APs of BEMG.

MATERIALS AND METHODS

Bacterial strains, *B. cereus* NCIM 2700 and *S. aureus* NCIM 2127 (ATCC 9144) were procured from National Chemical Laboratory, Pune.

Phosphatidylinositol was obtained from Sigma-Aidrich (USA). Protein molecular mass markers were from Genei (Bangalore, India). Other chemicals and reagents of AR grade were procured from E.Merck (India) or SD Fine.

Bovine blood was obtained from a veterinary hospital in Mumbai.

Purification of PIPLC: PIPLC was purified from *B. cereus* as described by Rastogi et al. (2005).

For purification of Sa-PIPLC, sterilized medium containing peptone, 60 g; glucose 3.0 g; beef extract, 9.0 g; NaCl, 6.0 g; Na_2HPO_4, 1.2 g; Na_2CO_3, 7.5 g; and water to a total volume of 3 litres; pH 6.0 was inoculated with 1.0% v/v of the seed culture of *S. aureus*. Incubation was carried out at 37°C for 18 h at 200 rpm (Kothekar and Dasgupta, 2013). Cell free extract was subjected to ammonium sulphate precipitation. Precipitate obtained with 40-80 % ammonium sulphate was dissolved in 0.01 M Tris-HCl buffer, pH 8.2 and subjected to dialysis against 2 mM Tris-HCl, pH 8.2. The dialysate (40-45 mg protein) was loaded on Mono-Q column equilibrated with 0.01 M Tris-HCl buffer, pH 8.2. The column was eluted with a linear gradient of 0-0.5 mM trisodium citrate (1 ml/min flow rate) in the same buffer with FPLC system. Active Mono-Q fractions were applied on Sephadex G-75 column, equilibrated and eluted with 0.01M Tris-HCl, pH 7.5 containing 0.05 M NaCl at 1 ml/min flow rate. PIPLC-active fractions were pooled and concentrated using Amicon ultra 15 centrifugal devise and stored at -20°C until used.

Purified Bc-PIPLC and Sa-PIPLC were tested for presence of protease activity on gelatin agar plate. No zones of clearance around the wells containing PI-PLCs indicated absence of protease activity.

PIPLC assay

The phospholipid estimation method described by Eryomin and Poznyakov (1989) was modified and adapted for estimating PIPLC activity. PI-PLC activ-

Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

ity was determined using phosphatidylinositol (PI) as substrate. 10 mM solution of PI was prepared in 0.05 % (v/v) Triton X-100 and subjected to sonication for 3 min. The reaction mixture consisting of 10 mM PI (200 µl), 0.1 mM Tris-maleate buffer pH 6.0 (200 µl) and enzyme extract (200 µl), was incubated at 37°C for 20 min. The reaction was terminated by adding 2.5 ml of chloroform:methanol:HCl (66:33:1) mixture, vortexed for a min and centrifuged at 10,000×g for 10 min. From the lower chloroform layer, 200 µl aliquot was withdrawn and subjected to phospholipid analysis by the VBR dye method (Eryomin and Poznyakov, 1989). A standard graph was plotted with 10-100 µg PI.

One unit of enzyme activity is the amount of enzyme that catalyses the hydrolysis of one micromole of substrate per minute at 37˚C.

Protein estimation
After each purification step, protein content was determined by Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

Preparation of bovine erythrocyte membrane ghost BEMG were prepared by the method of Taguchi *et al.* (1984).

PIPLC-mediated release of GPI-APs from BEMG 200 µl BEMG preparation (0.3 mg/ml) in 0.1 M Tris HCl, pH 7.4 was incubated with 200 µl Bc-PIPLC or Sa-PIPLC (4 U/ml) or a mixture of the two PI-PLCs (2 U/ml each) at 37°C for 90 min. A control without PI-PLC was maintained. Each reaction mixture was then centrifuged at 40,000×g for 20 min to separate the solubilised membrane proteins from the membrane-bound form. The resulting supernatants were subjected to SDS-PAGE.

SDS-PAGE of proteins released from BEMG by PI-PLCs
SDS-PAGE was performed as described by Laemmli (1970). Gels were 0.5 mm thick and contained 10 % polyacrylamide in the separating gel. Protein samples were mixed 1:5 with 5X sample buffer and heated to 95°C for 5 min prior to loading. Gels were electrophoresed at constant current of 40 miliampere at 4°C and stained with Coomassie brilliant blue R-250.

Result
The SDS-PAGE pattern of GPI-APs released from BEMG by Bc-PIPLC and Sa-PIPLC was studied. The electrophoretic profile (Figure 1) clearly demonstrated the difference in action of the two PI-PLCs. Proteins, 29, 68 and 100 kDa were released by PI-PLCs from both the sources (lane 2 and lane 4 of Figure 1). However, two proteins 42 and 65 kDa were released by Bc-PIPLC but not by Sa-PIPLC (lane 2 and lane 4). 40 kDa protein was found to be released by only Sa-PIPLC (lane 4 of Figure 1). Further, the combination of these two PI-PLCs showed a cumulative effect in releasing GPI-APs (lane 3 of Figure 1). There was no detectable spontaneous release of membrane proteins in the absence of PIPLC (lane 5 of Figure 1).

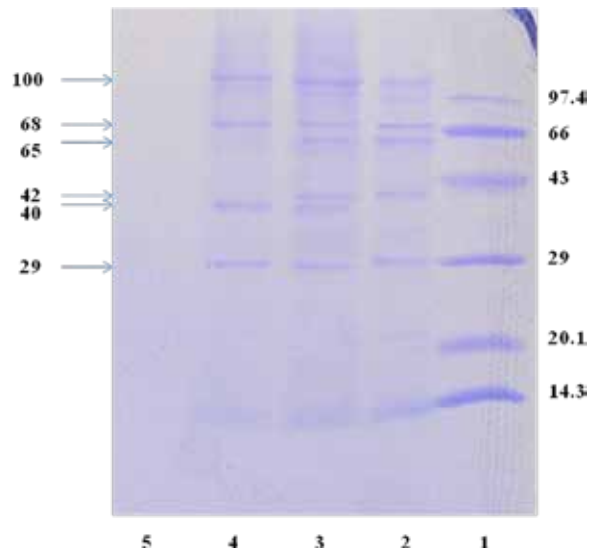

Fig. 1: Electrophoretic profile of proteins released from BEMG by PI-PLCs

Figures on either side indicate molecular mass in kilodalton. Figures at the bottom

indicate lane number. Lane 1: Marker proteins; Lane 2: Proteins released by Bc-PIPLC;

Lane 3: Proteins released by Bc- PIPLC + Sa-PIPLC ; Lane 4: Proteins released by Sa-PIPLC

Lane 5: BEMG supernatant without PI-PLC

* The result presented here is a representative of the experiment run in triplicate.

Discussion
In this study, the profile of GPI-APs released by the action of Bc-PIPLC and Sa-PIPLC on BEMG was compared. Molecular weights of the released proteins were 29, 40, 42, 65, 68, and 100 kDa (Figure 1). Of these membrane proteins, 30, 40 and 42 kDa proteins have been reported previously (Taguchi et al. 1999) and 68 kDa corresponds to acetylcholinesterase (Boschetii et al. 1996). The difference in the electrophoretic profile of the proteins released by Bc-PIPLC and Sa-PIPLC (Figure 1) implies that the membrane proteins differ in their resistance or susceptibility to the action of the two PIPLCs. The resistance of certain GPI-APs to PIPLC has been attributed to the structural variations found in the glycan bridge of GPI anchor (Ikezawa, 2002). This is supported by the observation that acyl substitution in the myo-inositol moiety makes the GPI anchor resistant to bacterial PI-PLC (Roberts et al., 1988). Another important factor that can influence the release of GPI-AP is the composition of the lipid environment of the membranes. Previous report on study with 5'nucleotidase has demonstrated that the efficiency of PI-PLC in releasing the membrane anchored protein varies with different bilayer lipid composition (Lehto and Sharom 1998). The relative surface charge of the plasma membrane, fluidity of the membrane and packing of membrane lipids have been suggested to be important modulators of bacterial PI-PLC activity on GPI anchors (Sharom 2010). The present study revealed that the resistance or susceptibility of GPI-APs to PI-PLC depends on the source of the enzyme. Although PIPLC from *B. cereus* and *S. aureus* show overall sequence

Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

homology, there are considerable differences in the amino acid composition of the two proteins, thus conferring distinct properties on the two enzymes (Kuppe et al. 1989, Daugherty and Low 1993). This can be the reason for the observed difference in the action of these two PIPLCs in releasing certain GPI-APs. This aspect should be taken into consideration while using PI-PLC as a molecular tool to identify GPI-APs. The results also suggest that a combination of PI-PLCs from two or more sources can be efficiently employed for identification of GPI-APs during membrane proteome studies than the conventional use of a single PI-PLC.

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